Insulin Receptors in Hepatocytes: Postreceptor Events Mediate Down Regulation

Abstract. *Down regulation of the insulin receptor of primary cultures of rat hepatocytes occurs in the presence of insulin and several agents with insulin-like activity, which act through or distal to the insulin receptor. These findings indicate that the interaction of insulin with its specific binding site is not in itself sufficient to down-regulate this receptor and that one or more steps subsequent to this interaction are necessary. Thus, down regulation may be a complex biological response to insulin, and if a cell were resistant to this effect of insulin, our data may explain how target cells from a patient or animal can have a normal number of receptors in the presence of increased concentrations of circulating insulin.*

That the number of insulin receptors in target tissues and the concentration of insulin in plasma are inversely related has been demonstrated in humans and animals in different metabolic states (*1*). Studies in vitro with IM-9 lymphoblastoid cells, human lymphocytes, rat adipocytes, and hepatocytes from regenerating rat liver have clearly shown an inverse relation between insulin and the concentration of its membrane receptors (*2*). However, there are notable exceptions (*3-6*) in which normal numbers of insulin receptors are associated with high concentrations of and resistance to insulin. These exceptions indicate that the mechanism of down regulation of the insulin receptor may be more complex than previously thought. The failure of some cells to down-regulate may depend on their resistance to the ability of insulin to down-regulate its receptor.

Self-regulation of the insulin receptor has been postulated to occur via the receptor itself largely by acceleration of receptor degradation (*2*). In the present studies we evaluated whether down regulation of the insulin receptor is directly attributable to the physical interaction of insulin with its receptor or is a postreceptor event.

We used primary cultures of isolated rat hepatocytes; such cells are well suited for binding studies and carry out numerous functions characteristic of the liver in vivo (*7*). Male Sprague-Dawley rats (150 to 250 g) fed ad libitum were used for liver cell isolation as previously reported (*8*). Primary cultures of isolated hepatocytes were prepared by the method of Kletzien (*7*) in serum-free medium. Any additions to the cultures were added at the time of the first medium change 4 hours after plating, at which time 37 percent ($\sim 2.5 \times 10^6$ cells per plate) of the cells had adhered to the plate as a monolayer. These primary cultures were then incubated for 16 hours in the presence of insulin ($10^{-7}M$) as well as substances that mimic insulin but do not interact with the insulin receptor [hydrogen peroxide (0.3 m*M*), spermine (0.3 m*M*), vitamin K_5 (0.05 m*M*)], a substance that mimics insulin and interacts with membrane glycoproteins [wheat germ agglutinin (10 μg/ml)], and antibodies to the insulin receptor (1/1000 dilution) (*9*). All of these agents mimic insulin action in fat cells (*9*) and freshly isolated rat hepatocytes (*10*). Table 1 shows that in primary cultures of hepatocytes at concentrations that have maximum effect, all these agents stimulated total lipid synthesis to the same degree as insulin, establishing their role as mimics of insulin in this cell system. Table 2 shows that when hepatocytes were cultured in the presence of insulin and the insulin mimics, there was a 46 to 61 percent decrease in the specific binding of tracer quantities of ^{125}I-labeled insulin ($1 \times 10^{-10}M$). Spermine down-regulated the insulin receptor in the presence of 3 g of albumin but not 0.1 g of albumin per 100 ml of medium. This is consistent with the findings of Livingston *et al.* (*11*) who reported that the insulin-like effects of spermine result from the formation of hydrogen peroxide during oxidative deamination of spermine by spermine oxidase, a known contaminant of fraction V bovine serum albumin.

Table 1. Effects of insulin and substances mimicking insulin on lipid synthesis in primary cultures of hepatocytes. Primary cultures of isolated rat hepatocytes were incubated in serum-free medium for 16 hours (*7*). Lipid synthesis was assessed in Hanks-Hepes buffer after washing the plates free of incubation medium. After 30 minutes of incubation in the absence and presence of insulin and the insulin mimics, [2-^{14}C]acetic acid (sodium salt, 5 m*M*, 0.01 mCi/mmole) was added to each plate. Thirty minutes later the medium was poured off, the plates were washed with cold phosphate buffer, and cellular lipids were extracted into a mixture of chloroform and methanol (*16*). Lipid synthesis was linear for 1 hour and more than 90 percent of the synthesized lipids were associated with the cells. The data represent the means (± standard error) of triplicate plates from three separate experiments.

Cell treatment	Lipid synthesis (pmole/min per plate)
Control	30 ± 1
Insulin ($1 \times 10^{-7}M$)	64 ± 1
Hydrogen peroxide (0.3 m*M*)	63 ± 3
Wheat germ agglutinin (10 μg/ml)	66 ± 3
Vitamin K_5 (0.05 m*M*)	65 ± 1
Insulin receptor antibodies (1/1000 dilution)	57 ± 6
Spermine (0.3 m*M*)	64 ± 2

Further studies were performed with insulin, insulin receptor antibodies, wheat germ agglutinin, and hydrogen peroxide to determine whether the changes in insulin binding are due to changes in the number of insulin receptors or the apparent affinity of this receptor for insulin and to establish the specificity of these effects. Figure 1 indicates that the decrease in insulin binding in the presence of insulin and insulin mimics is mainly due to a decrease in the number of insulin receptors without a change in the apparent affinity of the insulin receptor for insulin. When analyzed by linear regression of the high-affinity portion of the Scatchard plot (*12*), the dissociation constant (K_d) of binding is approximately $1 \times 10^{-9}M$ under all conditions.

We conducted further studies to determine whether down regulation by insulin and insulin mimics was a long-term effect unrelated to any interference of insulin or these agents with the binding of insulin. Before binding analyses were performed, cultures incubated overnight with insulin and the insulin mimics were washed twice at 37°C with Hanks-Hepes buffer containing glucose (8 m*M*) for two 30-minute periods at 37°C to allow dissociation of previously bound insulin. To test the adequacy of this washing procedure we incubated cells with $10^{-7}M$ unlabeled insulin at 37°C for 1 hour and washed them as described above. The binding of tracer quantitites of ^{125}I-labeled insulin ($10^{-10}M$) was 93 to 95 percent of that in cultures that had not been incubated with unlabeled insulin. The insulin mimics were washed similarly, but, in addition, wheat germ agglutinin was allowed to dissociate in the presence of *N*-acetyl-D-glucosamine (50 m*M*) (*13*). Furthermore, as shown in Fig. 1, Scatchard analyses of binding in the presence of hydrogen peroxide, wheat germ agglutinin, and the insulin receptor antibodies indicate that there is either no change in binding in the presence of hydrogen peroxide and the insulin receptor antibodies, or an increase in affinity in the presence of wheat germ agglutinin as previously reported (*13*).

Insulin degradation was analyzed by gel chromatography on Sephadex G-50 equilibrated and eluted with 4*M* urea,

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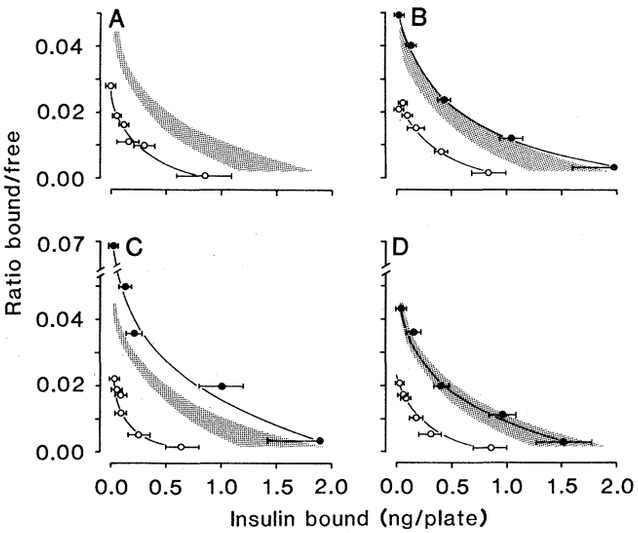

Fig. 1. Scatchard analyses of the insulin binding data. ^{125}I-Labeled insulin (1 Ci/μmole) was prepared by the chloramine-T method (*20*). Insulin binding was measured after 45 minutes of incubation at 25°C and expressed as specific binding, that is, the ^{125}I-labeled insulin ($1 \times 10^{-10}M$) bound after subtraction of the insulin remaining bound in the presence of $1 \times 10^{-6}M$ unlabeled hormone. Nonspecific binding was less than 10 percent under all experimental conditions. ^{125}I-Labeled insulin ($1 \times 10^{-10}M$) was incubated in the presence of increasing concentrations of native insulin in Hanks-Hepes buffer containing 8 mM glucose at *p*H 7.4. Cells were incubated for 16 hours in the absence of insulin and the presence of (A) insulin ($10^{-7}M$); (B) hydrogen peroxide (0.3 mM); (C) wheat germ agglutinin (10 μg/ml); or (D) insulin receptor antibodies (1/1000 dilution). For insulin, the culture medium was changed and insulin added at 6-hour intervals. Hydrogen peroxide was added at the same intervals. The serum containing the insulin receptor antibodies was heated at 56°C for 30 minutes to inactivate complement. Before binding was determined, the cells were washed as described in the text. The shaded area represents the mean (± standard error) of triplicate samples from six separate experiments in the absence of insulin or insulin mimics. Symbols: ○, mean (± standard error) of triplicate samples from three separate experiments in which the cells were treated for 16 hours; ●, mean (± standard error) of triplicate samples from three separate experiments in which ^{125}I-labeled insulin binding was assessed in the presence of the agents indicated in 16-hour cultures which had not been exposed to insulin or insulin mimics prior to the 45-minute incubation period with ^{125}I-labeled insulin.

1M acetic acid, and 0.1 percent Triton X-100 (*14*). Under the conditions used to assess insulin binding, and at a ^{125}I-labeled insulin concentration of $1 \times 10^{-10}M$, 88 percent of the radioactive material in the medium from control cells and 87 percent from treated cells eluted in the position of intact insulin. This demonstrates that the decrease in binding in treated cells is not due to an increase in insulin degradation. Insulin and the insulin mimics did not affect the specific binding of ^{125}I-labeled glucagon and ^{125}I-labeled wheat germ agglutinin (Table 2), suggesting that the down regulation induced by insulin and the insulin mimics is specific for the insulin receptor. Furthermore, the viability of control cells and treated cells was similar as assessed by light microscopy, by the concentration of protein per plate (Table 2), and by the incorporation of [^{14}C]leucine into proteins. The curve for the incorporation of [^{14}C]leucine was linear for at least 2 hours and was the same in all cell groups (Table 2).

Table 2. The effects of insulin and insulin mimics on hormone and wheat germ agglutinin binding and cell viability. We prepared primary cultures of isolated rat hepatocytes in serum-free medium by the method of Kletzien *et al.* (*7*). Hepatocytes were incubated for 16 hours in the absence or presence of insulin or insulin mimics as described in the legend to Fig. 1. Spermine was added every 6 hours as for hydrogen peroxide. At the end of the incubation period, cells were washed with Hanks-Hepes buffer as indicated in the text. Specific ^{125}I-labeled insulin binding was determined as indicated in the legend to Fig. 1. Specific ^{125}I-labeled glucagon binding (0.6 nM, 0.24 to 0.71 Ci/μmole), that is, the ^{125}I-labeled glucagon bound (0.6 nM) after subtraction of the glucagon that remained bound in the presence of 7.5 μM unlabeled hormone, was determined at 25°C after 45 minutes of incubation as previously reported (*17*). Specific binding of ^{125}I-labeled wheat germ agglutinin (26 ng/ml) was determined as that amount bound after 45 minutes of incubation at 25°C after subtraction of the wheat germ agglutinin that remained bound in the presence of *N*-acetyl-D-glucosamine, 50 mM (*13*). [^{14}C]Leucine (0.1 mg/ml, 0.3 μCi/ml) incorporation into proteins by the filter-paper disk method (*18*) was determined at 37°C and was linear for at least 2 hours. The data presented were calculated after 1 hour of incubation. The total amount of protein per plate was determined by the method of Miller (*19*). The numbers are means (± standard error) of triplicate samples from three different experiments.

Cell treatment	Specific binding of ^{125}I-labeled			[^{14}C]Leucine incorporated into proteins (count/min per plate)	Protein (μg/plate)
	Insulin (pg/plate)	Glucagon (fmole/plate)	Wheat germ agglutinin (ng/plate)		
Control	28 ± 1	50 ± 6	2.3 ± 0.1	166 ± 7	2617 ± 200
Insulin ($10^{-7}M$)	15 ± 1	41 ± 8	2.7 ± 0.1	184 ± 4	2647 ± 260
Wheat germ agglutinin (10 μg/ml)	15 ± 2	49 ± 5	2.2 ± 0.1	188 ± 4	2572 ± 320
Insulin receptor antibodies (1/1000 dilution)	14 ± 3	51 ± 6	2.6 ± 0.5	173 ± 4	2583 ± 130
Hydrogen peroxide (0.3 mM)	14 ± 1	52 ± 10	2.4 ± 0.3	188 ± 25	2628 ± 220
Vitamin K_5 (0.05 mM)	11 ± 1				
Spermine (0.3 mM)*	13 ± 3				
Spermine (0.3 mM)†	25 ± 6				

*In the presence of 3 g of albumin per 100 ml of medium. †In the presence of 0.1 g of albumin per 100 ml of medium.

It is widely held that for insulin to exert its biological effects it must first interact with the insulin receptor. The steps that follow this interaction and result in the known biological effects of insulin are largely unknown. Our studies indicate that the interaction of insulin with its specific binding site on the insulin receptor is not in itself sufficient to down-regulate the receptor and that one or more steps subsequent to this interaction are necessary. This is demonstrated by the ability of several agents that do not interact with the insulin receptor to specifically decrease the number of insulin binding sites. Although it is widely held that hydrogen peroxide, spermine, and vitamin K_5 mimic the effects of insulin through postreceptor mechanisms (*15*), the proximity of these events to insulin receptor binding is unknown. Our data suggest, however, that down regulation of the insulin receptor by insulin is a complex intracellular effect, and that resistance of cells to this effect may explain how a target cell from a patient or animal can have normal numbers of insulin receptors in the presence of increased concentrations of plasma insulin. For example, cells from some obese (*3*) and diabetic (*6*) subjects as well as from old rats (*5*) and Zucker rats (*4*) do not demonstrate down regulation in spite of the high plasma insulin concentrations.

JOSE F. CARO
JOHN M. AMATRUDA
Endocrine-Metabolism Unit, Department of Medicine, University of Rochester School of Medicine, Rochester, New York 14642

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Anticonvulsants Specific for Petit Mal Antagonize Epileptogenic Effect of Leucine Enkephalin

Abstract. *The anticonvulsants ethosuximide, sodium valproate, and trimethadione that are specific for petit mal epilepsy abolished in rats the electrical seizure activity and behavioral abnormalities produced by leucine enkephalin, whereas phenobarbital and phenytoin had no effect. The dose-response curve for naloxone against seizure activity induced by leucine enkephalin was the same as that in γ-hydroxybutyrate-induced petit mal. These data indicate that the epileptic properties of leucine enkephalin are petit mal–like and raise the possibility of involvement of enkephalinergic systems in petit mal epilepsy.*

Leucine enkephalin is a pentapeptide that occurs naturally in brain and is presumed to be one of the natural ligands for opiate receptor in brain (*1*). In 1977 Urca and colleagues (*2*) reported that methionine enkephalin, a natural ligand for the opiate receptor differing in its structure from leucine enkephalin only in the NH_2-terminal amino acid, produced epileptiform activity when introduced into the lateral ventricle of rats. Since then similar activity has been demonstrated for leucine enkephalin (*3*) and for β-endorphin (*4*), a larger opiate peptide. This paroxysmal activity can be aborted by the specific opiate antagonist naloxone, but not by other anticonvulsants used in generalized convulsive seizures (*4*). Conversely, naloxone has been ineffective in a number of generalized and partial seizure models in animals as well as complex partial seizure disorders in humans (*5*); however, we demonstrated that naloxone was effective in aborting petit mal seizures induced in animals by γ-hydroxybutyrate (*6*, *7*). We now report experiments on the efficacy of several anticonvulsants against seizure activity induced by leucine enkephalin in the rat. We found that the paroxysmal abnormalities produced by the leucine enkephalin were selectively aborted by anticonvulsants specific for petit mal. Furthermore, dose-response studies with naloxone showed that doses in excess of 4 mg/kg were required to block this action of leucine enkephalin.

We implanted epidural electrodes in male Sprague-Dawley rats (200 to 300 g) under pentobarbital anesthesia to record the electrocorticogram (ECoG). An indwelling cannula was also implanted stereotactically in the lateral ventricle. The ECoG was quantitated by an automated frequency analysis system based on the zero crossing method (*8*). Recordings were made with the animals moving freely in clear plexiglass chambers. Cannula position was confirmed histologically after completion of the experiments.

Animals were rested for 7 days after surgery. Then 100 μg of leucine enkephalin in 15 μl of lactated Ringer solution was injected intraventricularly while the ECoG was continuously monitored. Animals that showed a characteristic electrical response to the enkephalin were then assigned to either an anticonvulsant or a control group, and drug experiments were begun 7 days after the initial enkephalin injection.

The anticonvulsants used were ethosuximide, trimethadione, sodium valproate, diazepam, clonazepam, phenobarbital, and phenytoin. The dosages of each drug and the time elapsed from injection of the anticonvulsant drug to enkephalin administration are shown in Table 1. Control and drug-treated animals were paired for all experiments with the control receiving the vehicle of the drug. All drugs except leucine enkephalin were administered intraperitoneally. Once the anticonvulsant or vehicle was administered and the response to leucine enkephalin determined, the animal groups were interchanged, and the experiment was repeated after a 7-day rest period. We performed single and multiple dose anticonvulsant drug studies. For the multiple dose experiments, the anticonvulsant drugs or their vehicles were administered once daily for 7 days and the response to leucine enkephalin was determined; the animals were then rested for 1 week, control and drug-treated groups were interchanged, and the experiment was repeated. In addition to the anticonvulsant drug experiments outlined above, a dose-response curve was ascertained for naloxone against leucine enkephalin–induced seizure activity, with doses of naloxone ranging from 0.1 to 10 mg/kg.

Leucine enkephalin produced a consistent dramatic paroxysmal electrical response within the first 60 seconds of administration with changes lasting up to 6 minutes (Fig. 1). The animals were immobile during all these paroxysms and showed occasional myoclonic jerks. The enkephalin-induced paroxysms recorded on the ECoG were reflected in the frequency analysis system by an increase in the 3- to 6-Hz band.

In the single dose studies (Table 1) ethosuximide and sodium valproate completely prevented the electrical and behavioral responses to the enkephalin in all animals. Trimethadione was completely effective in abolishing responses in three animals and lessened the duration and voltage of the discharge in three. Diazepam was completely effective in three animals and ineffective in three animals. Phenobarbital and phenytoin had no effect on the activity; however, clonazepam changed the pattern of

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